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The TGF-β/SMAD signalling system and extracellular matrix changes in myocardial remodelling and reverse remodelling following correction of pressure overload

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ABBREVIATIONS

TGF-β	transforming growth factor-β
SMAD	small mothers against decapentaplegic
ECM	extracellular matrix
ALK	activin like kinase
SMURF	SMAD ubiquitin regulatory factors
SARA	smad anchor for receptor activation
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinases
GO	gene ontology
FDR	false discovery rate
QPCR	quantitative real time polymerase chain reaction
cDNA	complimentary DNA
ELISA	enzyme-linked immunosorbent assay
HPLC	high pressure liquid chromatography
SEM	standard error of the mean
GDF-15	growth differentiation factor-15
SERCA2	sarcoplasmic reticulum calcium ATPase 2

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LIST OF PAPERS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I **Johannes L. Bjørnstad, Nils O. Neverdal, Øystein A. Vengen, Cathrine W. Knudsen, Trygve Husebye, John Pepper, Mons Lie, Geir Christensen, Theis Tønnessen.** Alterations in circulating activin A, GDF-15, TGF-beta3 and MMP-2, -3, and -9 during one year of left ventricular reverse remodelling in patients operated for severe aortic stenosis. *Eur J Heart Fail* 2008;10:1201-1207.

II **Johannes L. Bjørnstad, Biljana Skrbic, Ivar Sjaastad, Geir Christensen, Theis Tønnessen.** Non-invasive evaluation of a mouse model of reverse cardiac remodelling following banding-debanding of the ascending aorta. S*ubmitted.*

III **Johannes L. Bjørnstad, Biljana Skrbic, Henriette S. Marstein, Almira Hasic, Ivar Sjaastad, William E. Louch, Geir Florholmen, Geir Christensen, Theis Tønnessen.** Inhibition of SMAD2 phosphorylation preserves cardiac function during pressure overload. S*ubmitted.*

IV **Johannes L. Bjørnstad, Ivar Sjaastad, Ståle Nygård, Almira Hasic, Mohammad Shakil Ahmed, Håvard Attramadal, Alexandra V. Finsen, Geir Christensen, Theis Tønnessen.** Collagen isoform shift during the early phase of reverse left ventricular remodelling after relief of pressure overload. *Eur Heart J 2010, in press.*

INTRODUCTION

Aortic stenosis is the most common aetiology for aortic valve replacement (1). Calcification of the valve is the usual aetiology (2) leading to stiffening of the leaflets and gradually increased constriction usually developing over decades. Pressure overload leads to left ventricular remodelling, typically with development of concentric hypertrophy. The initial phase of remodelling is considered to be a beneficial and compensatory process reducing wall-stress. Ultrastructurally there is hypertrophy of cardiomyocytes and increased myocardial fibrosis (3). A gene shift with increased expression of fetal genes such as α-skeletal actin, β-myosin heavy chain and transforming growth factor β (TGF-β) also takes place (4-7). Subsequently myocardial remodelling leads to reduced cardiac function (8) and eventually the patient develops clinical symptoms, which classically is angina, syncope or heart failure (9). In the later years also subtle symptoms, such as increased fatigability, have been recognized as symptoms of aortic stenosis (10). Unless operated, the prognosis after onset of symptoms is poor (Figure 1).

In aortic stenosis, a limited coronary vasodilator reserve in addition to increased left ventricular muscle mass may reduce the coronary blood flow per gram of muscle. This may cause subendocardial ischemia during increased cardiac work causing angina pectoris even

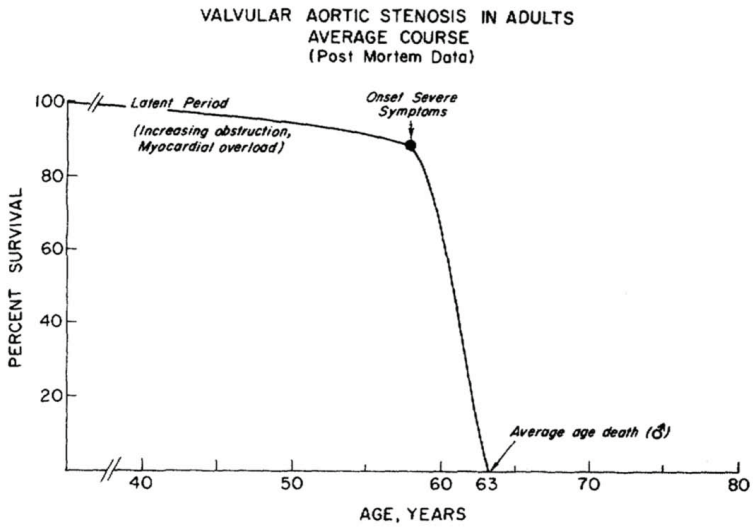


Figure 1. The natural history of aortic stenosis. The prognosis is poor following the onset of symptoms. *Modified after Ross J, Jr., Braunwald E. Aortic stenosis. Circulation 1968.*

in the absence of coronary artery disease (2).

Operative results for aortic valve replacement have improved over the years and mortality rates have declined to about 2% (11). The native aortic valve is replaced either by a mechanical valve or a biological valve prosthesis. While limited durability is the main concern for biological valves, the mechanical valves requires anticoagulation to avoid thromboembolic events. Although knowledge about myocardial remodelling is increasing, there are several crucial gaps in our understanding of this process, such as what causes the transition from compensated hypertrophy to overt heart failure and how the myocardial remodelling process is regulated. Correction of the increased afterload in aortic stenosis by aortic valve replacement leads to a process known as reverse remodelling. Left ventricular mass is usually normalized after 18 months (12, 13), while regression of fibrosis may take more than five years (14). There is limited knowledge regarding how the reverse remodelling process is regulated.

The heart may respond to hemodynamic stress by secreting different mediators (15, 16). Several growth factors and cytokines contribute to myocardial remodelling and may contribute to cardiac dysfunction (17) and a role for members of the TGF-β superfamily has been suggested (7, 18-22). The TGF-β superfamily is a large group of cytokines involved in several important biological processes, such as cell proliferation and differentiation in different tissues (vessels, skin), during embryonic development and in pathological processes such as fibrosis and cancer (23).

The SMAD (small mothers against decapentaplegic) proteins are transcription factors that are activated by different TGF-β superfamily members. The two main pathways are SMAD2/3 which is activated by TGF-β and activin, and SMAD1/5/8 which is activated by bone morphogenic protein (BMP) (Figure 2). Upon ligand stimulation TGF-β receptors type I and II form a complex leading to phosphorylation of SMADs. There are seven different type I receptors, activin like kinase (ALK) 1-7, with affinity to different SMADs. The SMADs form a complex with co-SMAD4, which translocates to the nucleus and regulates transcription (Figure 2) (24). There are two inhibitory SMADs, SMAD7 inhibits SMAD2/3 and SMAD6 inhibits SMAD1/5/8 (25). In addition there are interactions with different proteins, such as SMURF (SMAD ubiquitin regulatory factors) and SARA (Smad Anchor for Receptor Activation) (26). There is strong reason to believe that the TGF-β superfamily might play a role in cardiac disease (25).

The extracellular matrix (ECM) provides support and anchorage for cells. Collagen is an important ECM protein in the heart and is a major determinant of myocardial stiffness (27, 28). In addition to collagen, the ECM is composed of elastin, proteoglycans, hyaluronic acid, fibronectin and laminin. The ECM has been recognized to be more than an inert micro-

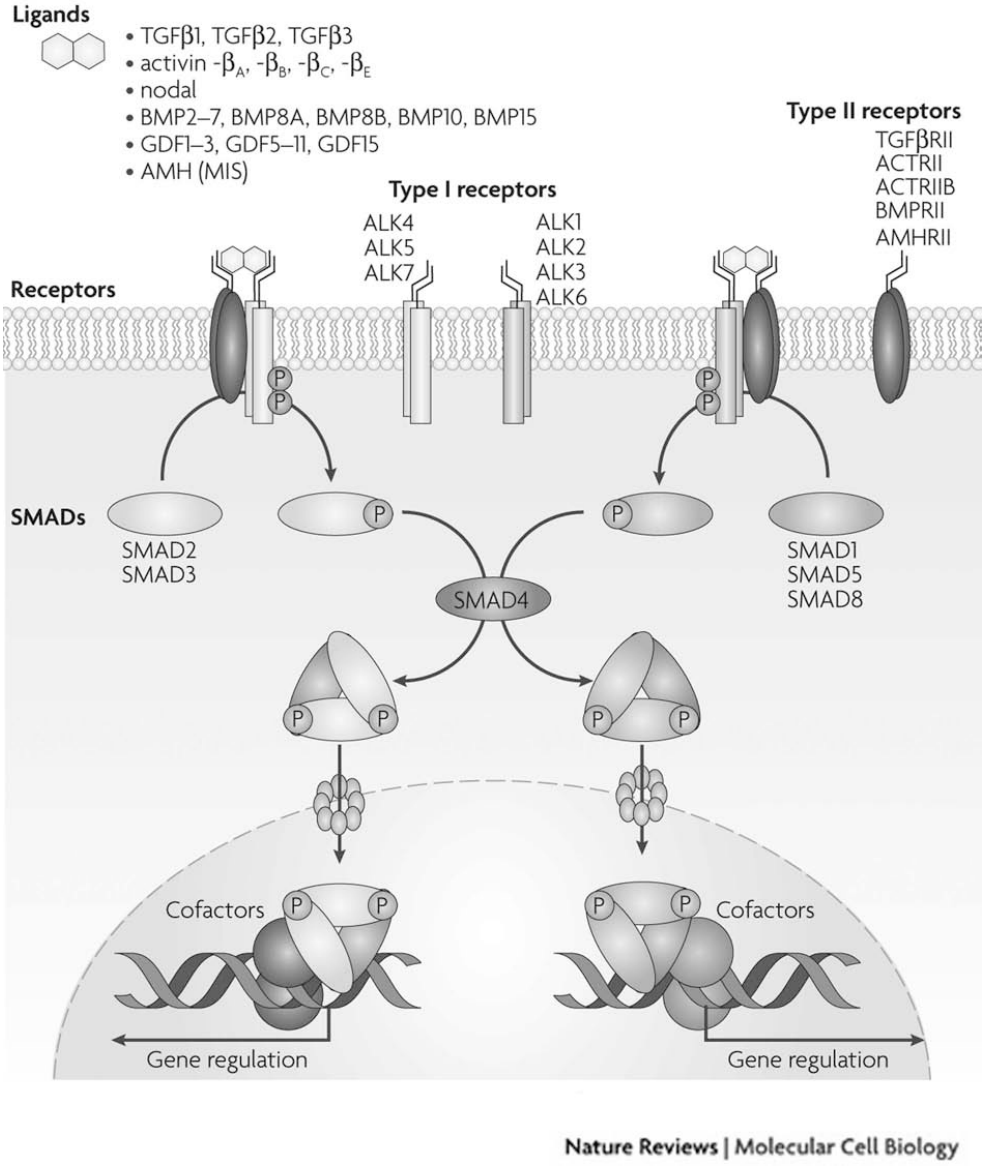


Figure 2. The SMAD signalling system. *Modified after Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol 2007.*

skeleton, as there are important interactions with the cells, e.g. acting as a reservoir for growth factors (29). Mature collagen is made up of three polypeptide strands that are twisted and coiled into a triple-helix. There are fibrillar and non-fibrillar collagens. Type I, II,

III, V and XI are fibrillar collagens (30). Type IV is important in the basal lamina (31). Type I and III are the most abundant collagens in the adult heart (32). Type I is also abundant in the Achilles tendon, it forms long fibers and provides tensile strength (33, 34). Extensile properties have been attributed to collagen type III, which is found in arteries and the uterus (35-37). Collagen VIII may form hexagonal networks (38). A total of 29 different collagen types have been identified so far. Also important for the ECM are the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). The MMPs are a group of zinc-dependent endopeptidases, which are capable of degrading ECM proteins including the collagens. There are four different TIMPs that act as key regulatory proteins by inhibiting MMP activity. There is increasing evidence that ECM changes are important in myocardial remodelling and that the MMPs and TIMPs have an important role in regulating ECM constitution (39).

The myocardial remodelling process has been addressed in numerous studies, however, whether TGF-β/SMAD signalling is beneficial or deleterious still remain to be resolved (40, 41). Furthermore, our knowledge about the reverse remodelling process taking place after correction of pressure overload, such as following aortic valve replacement for aortic stenosis, is limited. Thus, aortic stenosis is a common, serious disease leading to detrimental myocardial remodelling. Results from operative treatment are good, however extensive preoperative remodelling implies increased operative risk (42). Extensive remodelling may also cause persisting long-term cardiac dysfunction and incomplete reverse remodelling is associated with persisting cardiac dysfunction (12). Even though knowledge regarding the involvement of different mediators in myocardial remodelling is increasing, there are still gaps in our understanding of how the myocardial remodelling process is regulated. The reverse remodelling process has been less studied and we do not know whether there are mediators regulating this process. The TGF-β superfamily most likely plays a role in myocardial remodelling, however it has not been studied if the TGF-β superfamily plays a role in the regulation of reverse remodelling.

AIMS

The main aim of this thesis was to study the TGF-β superfamily and ECM changes in human and experimental aortic stenosis and during reverse remodelling following surgical correction of pressure overload.

PAPER I:

We wanted in paper I to examine plasma levels of activin A, GDF-15 and TGF-β3 and serum levels of MMP-2, MMP-3 and MMP-9 before and after aortic valve replacement for aortic stenosis. We furthermore wanted to correlate mediator levels to parameters of physical performance and left ventricular remodelling.

PAPER II:

The aim in paper II was to establish a hemodynamically well characterized mouse model of reversible left ventricular pressure overload by banding and subsequent debanding of the ascending aorta.

PAPER III:

In paper III we aimed to determine the role of SMAD2/3 signalling on myocardial remodelling and cardiac function following pressure overload using a pharmacological inhibitor of SMAD2/3 signalling in a mouse model of aortic banding.

PAPER IV:

In paper IV we wanted to examine alterations in myocardial gene expression and subsequently to identify molecular alterations important for the early phase of reverse remodelling following correction of pressure overload in a mouse model.

METHODOLOGICAL CONSIDERATIONS

ANIMAL MODELS

Although there are obvious major phenotype differences, such as size, tail, ears, fur and bilateral venae cavae superior, 99% of mouse genes have a detectable human homolog (43). There are also physiological differences, e.g. basal heart rate which is about 600-700 bpm in mice (44) and 60-70 bpm in man. Mice, however, are attractive for comparative medical research as mice are small, affordable and do not require much space in comparison with larger animals such as the pig. The mouse genome has been sequenced and transgenic and knock-out techniques have been developed (43). In addition most lab mice are inbred which reduces inter-individual variability, and in particular genetic variability, making mice the current ideal species for lab studies of gene expression (45). In the later years there have been several advances in surgical mouse models, such as aortic banding inducing left ventricular pressure overload mimicking aortic stenosis and hypertension, as well as in refinement of anaesthetic techniques (46). The development of methods for hemodynamic evaluation in mice, such as echocardiography, pressure-volume catheters and cardiac magnetic resonance imaging, has also been impressive (44). However, to be able to transfer scientific discoveries in mice models to knowledge in human medicine, the findings should at least be supported by observations in patients. We have established a banding-debanding

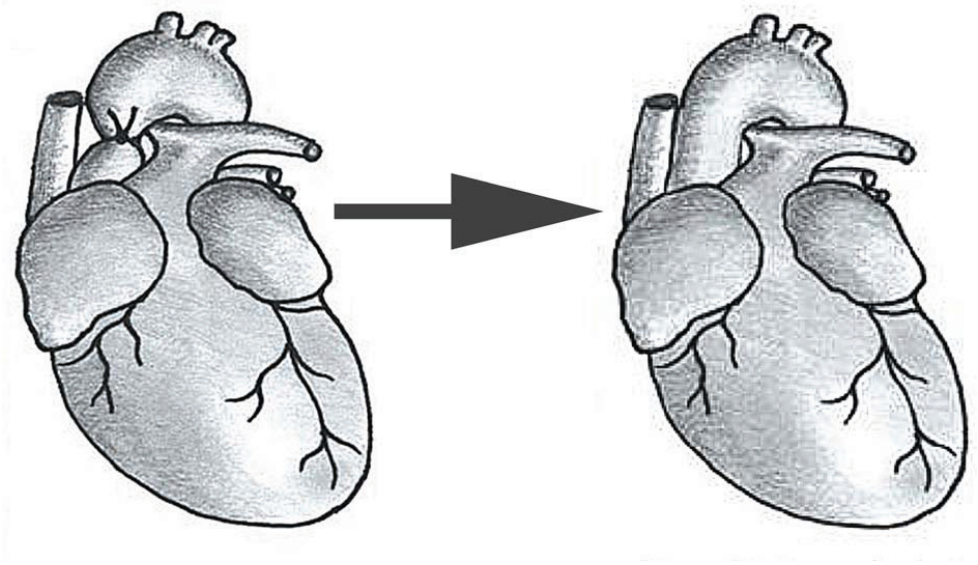


Figure 3. Reverse remodeling was induced in mice by banding and subsequent debanding of the ascending aorta. *Modified after Tarnavski O. Mouse surgical models in cardiovascular research. Methods Mol Biol 2009.*

model, in which mice are subjected to four weeks of banding of the ascending aorta, followed by a debanding operation (Figure 3).

ECHOCARDIOGRAPHY

Mouse echocardiography represents a great advance in the field of comparative cardiac research as it allows for in vivo non-invasive measurements of cardiac structure and function. The opportunity to perform repeated measurements in the same individual is an additional benefit. Spatial and temporal resolution is steadily improving and more refined techniques, such as tissue Doppler imaging and strain echocardiography have been developed. However, there are several concerns to be addressed in mouse echocardiography. The collection of echocardiographic recordings is a delicate skill (47) and all recordings in this thesis have been performed by one operator. The operator must be unbiased and should preferably be blinded from treatment groups. If echocardiography is to be preformed in wake animals the animals should be acclimatized to the examination situation by daily training to reduce stress. If animals are sedated care should be taken to avoid deep sedation as this leads to cardiodepression. The level of sedation should also be carefully standardized to avoid excess variability that might mask important findings. Anesthetized mice rapidly lose body heat and hypothermia reduces cardiac function, thus, a heat source should be used and body temperature should preferably be monitored. A swift recording procedure by a trained operator enhances the quality of data from mouse echocardiography.

Most of these concerns are not valid for echocardiography in human patients, as this examination usually is performed while the patients are awake, the human heart is larger and the heart rate is slower which allows for better resolution. However standardized recordings and measurements performed by an unbiased and experienced echocardiographer is critical for good data quality and the examiner should preferably be blinded to patient details.

MICROARRAY SCREENING

Microarray screening is a recent technological development which allows for simultaneous analysis of expression of multiple genes. Affymetrix GeneChip analyses over 39.000 transcripts on one single array. The processing of microarray data has also been refined and open source software is shared in the scientific community through BioConductor (http://www.bioconductor.org/). Bioinformatics' software, such as topGO, allows for elegant analyses and interpretation of microarray data. Biological function can be difficult to

understand from regulation of single genes and by using a refined bioinformatics algorithm TopGO scores functional gene ontology (GO) groups taking local dependencies between GO terms into account (48). The substantial amount of data generated from microarray screening, however, may be confusing and difficult to interpret. The large number of analyses also represents a statistical concern, as a level of significance of $p = 0.05$ just by chance would generate 5 positive tests out of 100 performed. Thus, 39.000 tests would by chance generate 1950 positive tests. There are statistical methods to correct for multiple testing, such as the Bonferroni correction. However, a rigid method to correct for multiple testing will disguise potential important findings in such a screening. False discovery rate (FDR) is the expected rate of false positive results in an analysis of multiple genes and statistical methods to estimate FDR have been developed and represent important tools in the analysis of microarray data (49). We have therefore collaborated closely with statistical experts in the field of bioinformatics'. The cost of gene chips is rather high, which usually leads to a rather small number of animals included in microarray studies. Important findings must be verified either on the transcriptional level (e.g. by QPCR) or by protein analyses (see below).

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

Quantitative real time polymerase chain reaction (QPCR) is a method to analyze expression of individual genes, by determining levels of mRNA with a particular genetic sequence in a specimen (50-52). This technique is now widely used to quantify mRNA. Following mRNA isolation reverse transcriptase is used to create complimentary DNA (cDNA), as mRNA is an unstable molecule. On sacrifice of the animal, care should be taken to snap freeze tissue in liquid nitrogen (-80°C) without delay, as mRNA degradation may introduce variability in the data from QPCR analyses that might be avoided. cDNA is amplified by reaction with a fluorescent reporter and measured in a thermocycler. The cycle at which the fluorescence crosses the threshold is determined and this corresponds to the level of the mRNA level in the specimen. A small amount of tissue yields sufficient mRNA to perform multiple QPCR analyses. QPCR is a sensitive method to detect transcriptional alterations. As it is less laborious than, for example, Western blot, QPCR is an attractive method for verifying microarray findings.

WESTERN BLOTTING

While an analysis of gene expression may be used as a marker of protein synthesis, Western blot (immunoblot) is an analytic technique to determine protein levels (53, 54). Briefly, proteins are isolated from tissue specimens, separated by size using gel electrophoresis,

transferred to a membrane and detected using specific antibodies, visualized by chemiluminescence and quantified by densitometry. Protein loading may be controlled by Commassie Blue staining. Antibody qualities are most important if false negative and false positive results are to be avoided. Another limitation is the number of lanes in each gel (15 in our lab). We have in our experiments used a reference group (sham aortic banding mice fed standard chow) in each gel which makes it possible to make comparisons across multiple gels.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assay (ELISA) is another technique for protein analyses (55, 56). We have used commercial kits using the "sandwich ELISA" technique. Briefly the kit contains a microplate pre-coated with antibody specific for the protein to be analyzed. Standards and samples are applied and unbound substances are washed away. An enzyme linked protein specific antibody is added to the wells, the plate is washed and a solution with a chemical being converted to colour by the enzyme is applied. The intensity of the colour is measured. The intensity of each sample is related to the standard curve which gives the concentration of the protein of interest in the specimens. Antibody specificity is of course important also in this technique.

MULTIPLEX

A multiplex protein assay allows for simultaneous examination of multiple proteins in a sample (57, 58). This is particularly useful when the biological sample is of limited amount. The technology is based on a family of fluorescently dyed microspheres, or beads, from Luminex Corp., the use of a special flow cytometer to measure the reactions occurring on the surface of the microspheres and a high-speed digital signal processor to manage the fluorescent output. Antibody specificity is of high importance in this technique. While proteins are separated by size in Western blot, this is not the case with multiplex and ELISA analyses, thus antibody specificity is even more important for both these techniques than for Western blot.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

High pressure liquid chromatography (HPLC) is a biochemical technique to quantify the concentration of different molecules (59). Briefly the sample to be analyzed passes through a column and the analyte is slowed from physical and chemical interaction with the column.

Thus, the time it takes a particular analyte to pass through the column is a fairly specific property of the substance to be quantified. Finally, the analyte is detected by fluorescence.

CALCIUM TRANSIENTS

Cardiomyocytes were isolated from the excised heart (60). Briefly, the heart was perfused with collagenase in a Langendorff setup. Collagenase breaks down collagen, which is a key component in the extracellular matrix. A biopsy was minced and gently shaken in a solution, filtrated and sedimented. The isolated cells were then washed from the enzyme solution. Aortic cannulation can be challenging in mice subjected to ascending aortic banding and care should be taken during the banding operation to place the ligature cranially on the ascending aorta. Myocytes were plated in an open-perfusion chamber mounted on the stage of an inverted microscope, labeled with fluo-4 AM and perfused with Hepes Tyrode's solution. Ca^{2+} transients were calculated from whole cell fluorescence recorded during field stimulation at 1 Hz under a mercury lamp (60-62). Isolated cardiomyocytes are sensitive to temperature and care must be taken to maintain ambient temperature at 37 °C during the experiments.

CARDIOMYOCYTE CULTURE

Cardiomyocytes were isolated from neonatal Wistar rats (1-3 days old) (63). Briefly, hearts were cut into small pieces and digested with collagenase and pancreatin. To separate cardiomyocytes from non-cardiomyocytes, the cell suspension was centrifuged through a discontinuous Percoll gradient. Cardiomyocytes were allowed to attach to culture dishes overnight in plating medium. Stimulation with TGF-β1 and SM16 was initiated following 24 h of serum starvation. Cells need stable conditions both with respect to temperature (37 °C) and medium.

PHARMACOLOGIC INHIBITION BY SM16

SM16 is a novel small molecule inhibitor of TGF-β type I receptor, which was generously provided by Dr. Leona Ling at Biogen Idec (64). In vitro efficacy of SM16 has been tested in cultured human liver carcinoma cells (HepG2) (64) and murine AB12 tumor cells (65) and inhibition of SMAD2 phosphorylation has been demonstrated at 620 nmol/L. Its specificity has been tested against 60 different kinases and SM16 effectively inhibits ALK 5 and ALK4, without effect on ALK1 or ALK6. Weak inhibitory effect has been demonstrated against P38/SAPK2a and Raf. Routes of administration and in vivo dose-response have been

determined in BALB/C mice and efficacy of orally administrated SM16 was excellent (65). SM16 formulated into murine chow at a dose of 0.45g SM16/kg chow led to a plasma concentration of 6.7±2.4 µmol/L (Figure 4). Plasma levels were determined in blood samples from a.m. and p.m. time-points without significant differences being described (65). At 0.25, 0.45 and 0.65 g SM16/kg chow no toxic effects were observed, but at the highest dose (1.7 g SM16/kg chow) the animals suffered from weight loss and ill-looking appearance most likely demonstrating a toxic effect at high doses. Thus, we were recommended the dose of 0.45 g SM16/kg chow by our collaborator at Biogen Idec.

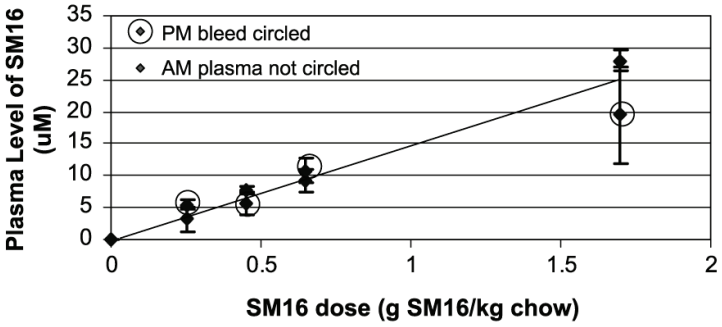


Figure 4. In vivo dose response of SM16 in mice. *Modified after Suzuki E et al. A novel small-molecule inhibitor of transforming growth factor beta type I receptor kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection. Cancer Res 2007.* Afternoon (PM), early morning (AM).

STATISTICS

Data are presented as mean±standard error of the mean (SEM). Data not normally distributed were logarithmically transformed. Parametric statistics were used where data conformed to the assumptions of normality and non-parametric statistics when this criterion was not fulfilled. In paper I and for echocardiographic measurements in paper IV one way repeated measures ANOVA, Friedman Repeated Measures ANOVA on Ranks, two-sided paired t-test or Wilcoxon Signed Rank Test was used to analyze repeated measurements from the same individual. When comparing two different groups two-sided Student's t-test, Welch t-test or Mann–Whitney Rank Sum Test was used. In paper III, data were analyzed by standard two-way ANOVA, as this takes into account the two different interventions in this model (pressure overload and SM16 treatment). In paper IV, we employed an ANOVA model for each gene that was programmed in R-script. This allowed for identification of the effects

of correcting pressure overload, taking into account the effects of surgical trauma and pressure overload itself. The relation between two continuous variables was examined by linear regression. Multiple comparisons were corrected for by Dunn's test for non-parametric and the Holm–Sidak method for parametric tests. For microarray data multiple comparisons were corrected for by using FDR according to Benjamini & Hochberg (49). We used $p \leq 0.05$ as the level of statistical significance. The R Project for Statistical Computing or SigmaPlot was used for the analyses.

SUMMARY OF RESULTS

PAPER I:

Twenty-two patients undergoing aortic valve replacement for aortic stenosis were examined by echocardiography and a six minute walking test preoperatively, 2 days, 6 and 12 months postoperatively. Plasma levels of activin A, growth differentiation factor-15 (GDF-15) and TGF-β3 and serum levels of MMP-2, -3 and -9 were analyzed by ELISA. Preoperatively the average peak valvular gradient was 89.3±4.8 mmHg, indicating severe aortic stenosis. Aortic valve replacement induces reverse remodelling and left ventricular mass was reduced from 198±14 g/m^2 preoperatively to 120±7 g/m^2 after 12 months. Left ventricular ejection fraction was normal and was unaltered in this study, however, there was a reduction in NYHA class from 2.3 to 1.3, and six minutes walking test was increased from 384±18 to 408±15 m, indicating improved physical capacity. Activin A was increased in patients with aortic stenosis compared to healthy controls, increasing further at 6 and 12 months following aortic valve replacement and was associated with reduced physical performance (Figure 5). GDF-15, MMP-3 and -9, for which effects on fibrosis and hypertrophy have been reported, were increased 2 days postoperatively. MMP-3 correlated positively to left ventricular end diastolic diameter at 6 and 12 months postoperatively (R = 0.64 and 0.51, respectively). GDF-15 correlated positively to MMP-3 preoperatively and 2 days postoperatively (R = 0.43 for both).

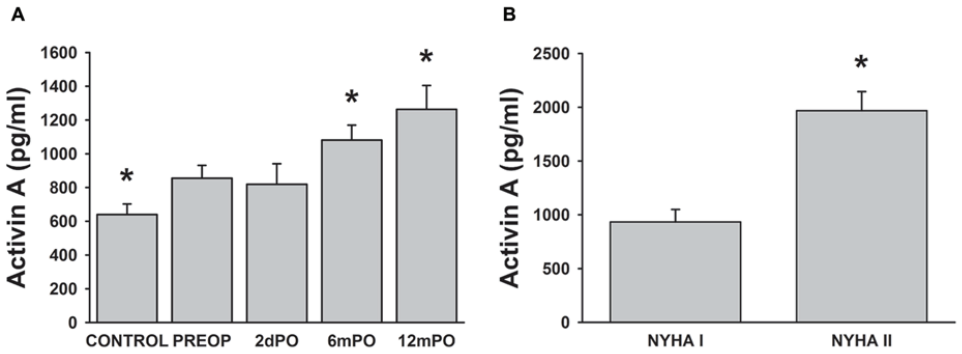


Figure 5. Activin A was increased in patients with severe AS and increased further following AVR (A). At 12 months postoperatively activin A was higher in patients with residual symptoms (B). Data are presented as mean+SEM. **A.** Preoperatively (PREOP); 2 days postoperatively (2dPO); 6 months postoperatively (6mPO); 12 months postoperatively (12mPO). * $p \leq 0.05$ vs PREOP. **B.** * $p=0.001$ vs NYHA I, n=22. *Adapted from paper I.*

PAPER II:

To be able to study reverse remodelling following correction of pressure overload, we wanted to establish a mouse model with reversible left ventricular pressure overload by banding and subsequent debanding of the ascending aorta. Furthermore, there was a need for careful hemodynamic evaluation and serial analyses of left ventricular mass as a marker of myocardial remodelling and reverse remodelling. Paper II describes our banding-debanding mouse model in detail. We found four weeks banding of the ascending aorta to induce concentric left ventricular remodelling, as left ventricular weight was increased by 59% and left ventricular wall thickness was increased with respect to chamber diameter. On average, lung weight was 70% increased. Elevated left ventricular filling pressure was suggested by increased left atrial diameter (47), indicative of pulmonary congestion. Echocardiography demonstrated reversible myocardial dysfunction as evaluated by systolic and diastolic tissue velocities.

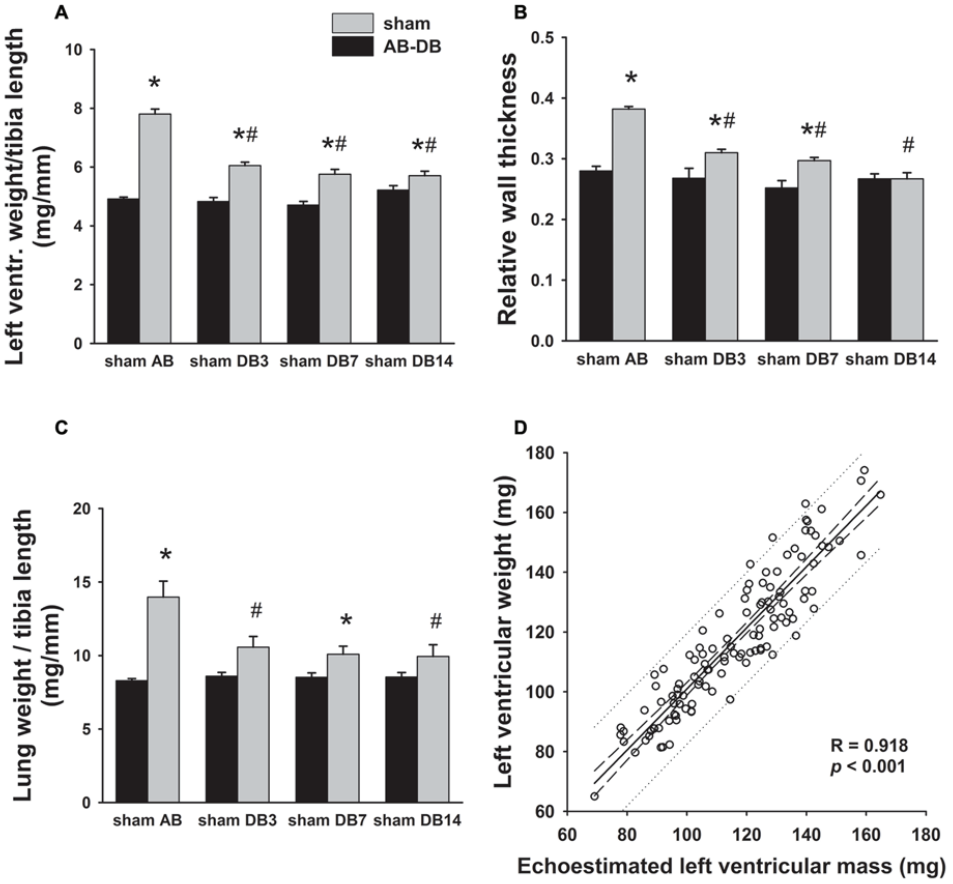


Figure 6. Aortic banding induced concentric left ventricular remodeling, with reverse remodeling following debanding (A-B). Lung weight was increased following AB, with regression taking place following DB (C). Correlation between left ventricular mass estimated by echocardiography and left ventricular weight (D). **A-C.** Data are presented as mean+SEM. * $p \leq 0.05$ vs sham; # $p \leq 0.05$ vs AB (A: n=8-28, B: n=5-108, C: n=8-28). Aortic banding (AB); debanding (DB). *Adapted from paper II.*

PAPER III:

SMAD2/3 mediates TGF-β signalling intracellularly. To study the effects of SMAD2/3 signalling in pressure overload we used the novel SMAD2/3 inhibitor SM16 in mice subjected to aortic banding. Aortic banding led to increased SMAD2 phosphorylation and reduced cardiac function, as was demonstrated by pulmonary congestion and echocardiographic measurements. SM16 inhibited phosphorylation of SMAD2, improved cardiac function and attenuated the fetal gene shift (Figure 7). Aortic banding induced cardiomyocyte hypertrophy, with 38% increased cell width, 11% increased cell length and 58% increased cell area, which was inhibited by SM16. After one week of aortic banding myocardial collagen protein levels were not increased, hence we were not able to evaluate possible effects of SMAD2 on cardiac fibrosis. Left ventricular sarcoplasmic reticulum calcium ATPase 2 (SERCA2) was reduced following aortic banding, while SM16 treatment preserved the levels of this important calcium handling protein. SERCA2 levels correlated inversely to lung weight, indicating an association between reduced SERCA2 levels and pulmonary congestion. Furthermore, in cardiomyocytes isolated from banded mice, cytosolic Ca^{2+} removal was enhanced by SM16. A role for TGF-β/SMAD signalling in regulating SERCA2 was supported by *in vitro* studies of neonatal rat cardiomyocytes, as TGF-β1 induced phosphorylation of SMAD2 led to reduced SERCA2 mRNA expression which was prevented by SM16 (Figure 7).

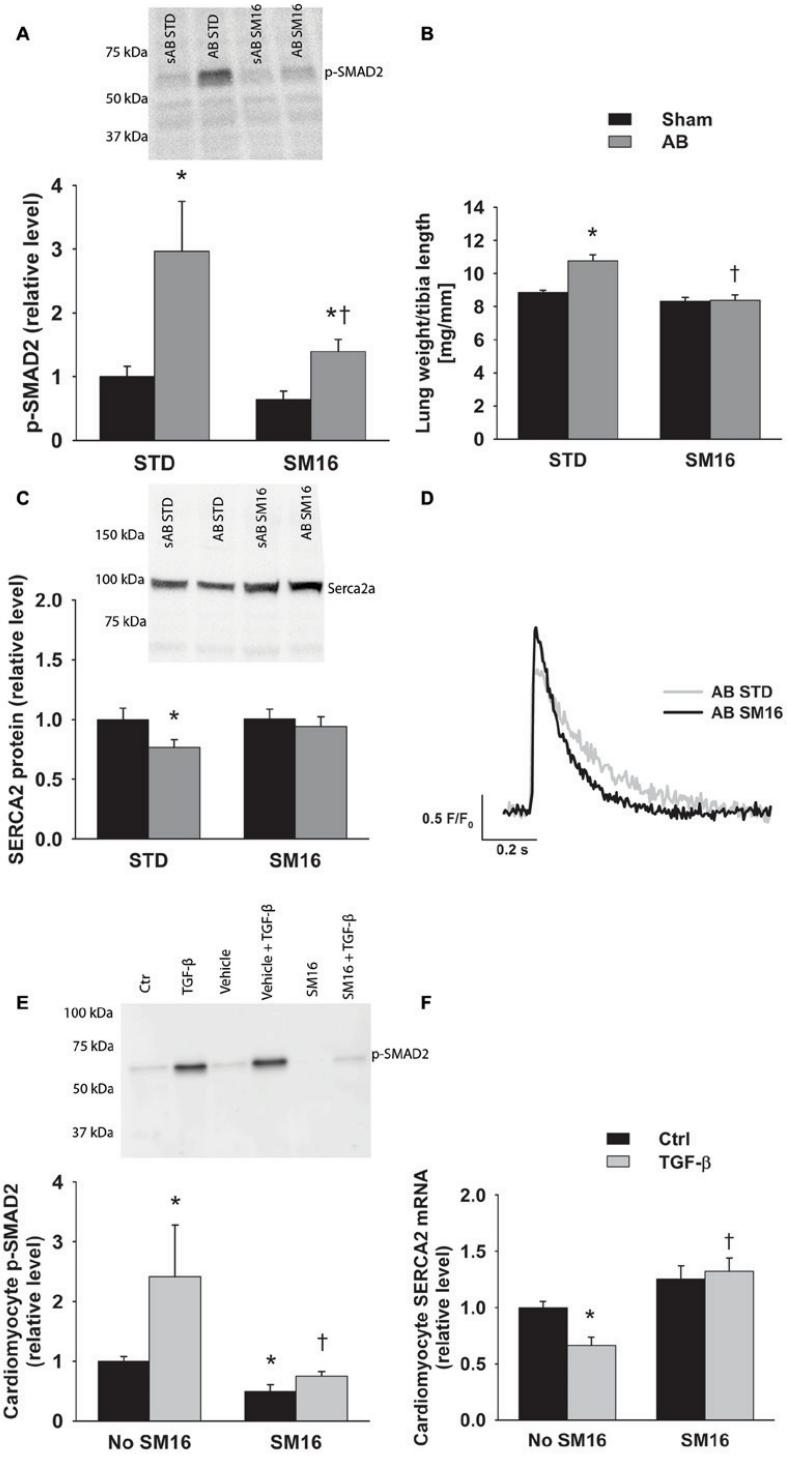
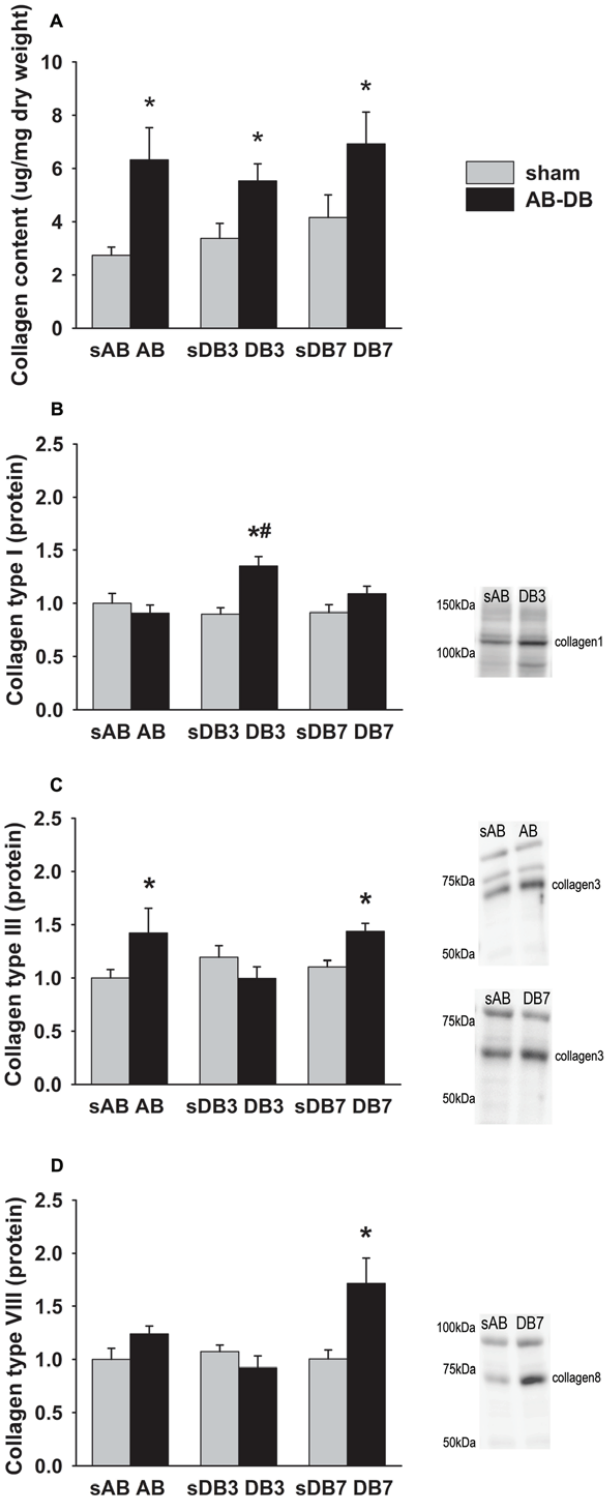

A
sAB STD
AB STD
sAB SM16
AB SM16
75 kDa
50 kDa
37 kDa
p-SMAD2
p-SMAD2 (relative level)
STD
SM16
B
Sham
AB
Lung weight/tibia length [mg/mm]
STD
SM16
C
150 kDa
100 kDa
75 kDa
Serca2a
SERCA2 protein (relative level)
STD
SM16
D
AB STD
AB SM16
0.5 F/F0
0.2 s
E
Ctr
TGF-β
Vehicle
Vehicle + TGF-β
SM16
SM16 + TGF-β
100 kDa
75 kDa
50 kDa
37 kDa
p-SMAD2
Cardiomyocyte p-SMAD2 (relative level)
No SM16
SM16
F
Ctrl
TGF-β
Cardiomyocyte SERCA2 mRNA (relative level)
No SM16
SM16

Figure 7. Aortic banding (AB) induced phosphorylation of SMAD2, which was inhibited by SM16 (A). SM16 treated animals did not develop increased lung weight following AB (B). SERCA2 levels were preserved by SM16 (C). SM16 improved Ca^{2+} handling by enhancement of transient decay (D). TGF-β1 stimulation of neonatal rat cardiomyocytes induced phosphorylation of SMAD2 which was attenuated by SM16 (E) and reduced SERCA2 mRNA expression which was prevented by SM16 (F). **A+C.** Data show mean relative intensity+SEM and representative blots. Sham STD=1. **B.** Data show mean+SEM. * $p \leq 0.05$ vs sham; † $p \leq 0.05$ vs STD (A: n=4-8, B: n=22-40, C: n=9-16). **D.** Representative figure of fluorescence intensity (F/F_0) by time (n=11-16). Aortic banding (AB); standard chow (STD); SM16 chow (SM16). **E.** Data show mean relative intensity+SEM and representativ blot. **F.** Data show mean relative mRNA expression+SEM. Ctrl+no SM16=1. * $p \leq 0.05$ vs ctrl, † $p \leq 0.05$ vs no SM16 (n=4-6). Control (Ctrl); stimulated with TGF-β1 (TGF-β); stimulated with SM16 (SM16). *Adapted from paper III.*

PAPER IV:

To study the reverse remodelling process, we used a mouse model of reversible pressure overload by ascending aortic banding and subsequent debanding, which is described in detail in paper II. To avoid possible confounding effects of heart failure, we included in this study only mice without signs of pulmonary congestion, as was defined by left atrial diameter < 2.2 mm after four weeks of banding. This corresponded to 50% increased lung weight with respect to sham and affected approximately 50% of the mice. In addition to concentric left ventricular remodelling, α-skeletal actin and β-myosin heavy chain mRNA expression were 6 to 8-fold increased following aortic banding, supporting significant myocardial remodelling. Surgical correction of left ventricular pressure overload induced reverse remodelling with a rapid decline in left ventricular mass during the first week after debanding. Left ventricular collagen, however, remained increased. A functional analysis of the results from microarray screening disclosed that genes related to the extracellular matrix, and in particular the collagen genes, were the ones most strongly regulated from correction of pressure overload. This finding led us to identify a novel myocardial collagen isoform shift during the early phase of reverse remodelling. While total collagen remained 2-fold increased with respect to sham, there was a change in collagen isoform constitution. Following aortic banding collagen type III was most increased, at 3 days after debanding type I was increased by 50%, while at 7 days type VIII was increased by 70% (Figure 8). We were not able to demonstrate increased cardiac synthesis of activin A, TGF-β or GDF-15 following debanding.

Figure 8. While total collagen remained increased (A), there was an isoform shift from type III, to type I and VIII (B-D) during the early phase of reverse remodeling. A. Total myocardial collagen from hydroxyproline content, μg/mg dry weight, mean + SEM. * $p \leq 0.05$ vs. sham (n=9-10). B-D. Western blot of collagen subtypes. Sham AB was set to 1. Data show mean relative intensity + SEM and representative blots. * $p \leq 0.05$ vs. sham, # $p \leq 0.01$ vs. AB (n=5-9). Aortic banding (AB); debanding (DB); sham aortic banding (sAB); sham debanding (sDB). *Adapted from paper IV.*


A
Collagen content (ug/mg dry weight)
10
8
6
4
2
0
*
*
*
sham
AB-DB
sAB AB
sDB3 DB3
sDB7 DB7
B
Collagen type I (protein)
2.5
2.0
1.5
1.0
0.5
0.0
*#
sAB AB
sDB3 DB3
sDB7 DB7
150kDa
100kDa
sAB
DB3
collagen1
C
Collagen type III (protein)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
sAB AB
sDB3 DB3
sDB7 DB7
sAB
AB
75kDa
50kDa
collagen3
sAB
DB7
75kDa
50kDa
collagen3
D
Collagen type VIII (protein)
2.5
2.0
1.5
1.0
0.5
0.0
*
sAB AB
sDB3 DB3
sDB7 DB7
100kDa
75kDa
50kDa
sAB
DB7
collagen8

DISCUSSION

In paper I we have identified alterations in activin A and GDF-15 in patients with "reversible pressure overload" of the left ventricle undergoing aortic valve replacement for aortic stenosis. Activin A, GDF-15 and TGF-β activate the SMAD2/3 signalling system and share this as an intracellular second messenger. Both deleterious and cardioprotective effects have been attributed to this signalling system (19, 25, 66), which in paper III led us to study the effects of pharmacological inhibition of SMAD2/3 in left ventricular pressure overload. We found that the novel drug SM16 attenuated phosphorylation of SMAD2 and preserved cardiac function. We suggest beneficial effects on cardiomyocyte Ca^{2+} handling as the possible mechanism for this cardioprotective effect. In paper II, to mimic aortic valve replacement for aortic stenosis, we have established a mouse model of reversible left ventricular pressure overload by aortic banding and subsequent debanding of the ascending aorta. In paper IV, we found that the expression of genes regulating the ECM were the ones most altered following debanding. This led to the discovery of a novel collagen isoform shift during the early phase of reverse remodelling. We were not able, however, to identify increased cardiac synthesis of TGF-β superfamily members during reverse remodelling in mice.

COMPARATIVE MEDICINE

Exploring biological mechanisms in human studies may be difficult. Sampling of myocardial tissue is, for obvious ethical reasons, limited to a number of conditions, such as explanted hearts, donor hearts and small biopsies obtained during open heart surgery. Animal experiments allows for access to myocardial tissue for lab experiments. A number of animal models of heart disease have been developed. When designing an experimental study several issues should be taken into consideration. Pigs and humans are developmentally closely related and experimental studies in pigs have provided extensive physiological knowledge. In the later years, however, mice have grown popular in cardiac research. Despite obvious phenotype differences between mouse and man, gene homology is remarkable. In inbred mice, the genetic variation is extremely limited making this species excellent for studies of gene expression (67). The advances in genetically modified mice represent a powerful tool for researchers (43). During the last two decades several mouse models of human cardiac disease have been developed, among them is aortic banding, subjecting the left ventricle to pressure overload mimicking aortic stenosis and hypertension (46). Refined methods for hemodynamic evaluation in mice, such as echocardiography, have been established (68, 69). Low costs increase the feasibility of mice studies. Caution must be taken, however, when transferring the results from mice studies to human medicine and results should be supported at least by observational studies in man.

TRANSFORMING GROWTH FACTOR-β SIGNALLING

The TGF-β superfamily is a group of signalling proteins involved in several important biologic processes (25, 70). TGF-β/activin and BMP are considered as the two main groups of TGF-β superfamily members (25, 70). In addition to intracellular SMAD phosphorylation, non-SMAD pathways may also be activated (71). TGF-β and activin are ligands for ALK4 and 5 and ligand binding leads to SMAD phosphorylation. There is evidence that this signalling system leads to increased collagen synthesis (72-74). Involvement of TGF-β/SMAD signalling in cardiac disease has been suggested (25, 75) and TGF-β antagonism in pressure overload has attenuated myocardial fibrosis, however, both improved (41) and reduced (40) cardiac function have been reported. Kuwahara administered an anti-TGF-β antibody to rats undergoing suprarenal aortic banding and found reduced myocardial fibrosis and improved diastolic function (41). Lucas used a genetically modified mouse strain, with an inducible dominant negative mutation of the TGF-receptor type II which was unable to activate SMAD. Clear effects on cardiac remodelling were demonstrated as transverse aortic constriction in animals with impaired TGF-β/SMAD signalling developed left ventricular dilatation and reduced interstitial collagen and cardiac dysfunction was suggested (40). In paper I we demonstrated increased levels of activin A in patients with aortic stenosis. Following aortic valve replacement there was a further increase at 6 and 12 months, which represents an intermediate time-point during reverse remodelling, when left ventricular mass usually has normalized, but ECM changes persists. In addition there was an early increase of GDF-15 at 2 days postoperatively. In paper IV, however, we were not able to demonstrate increased cardiac synthesis of TGF-β superfamily members during reverse remodelling in mice. Species difference might be an explanation. The early changes described in paper I at two days postoperatively might be due to the surgical trauma and it is possible that the increased levels of activin A might represent an epiphenomenon, unrelated to reverse remodelling of the heart.

SMAD2/3 SIGNALLING IN PRESSURE OVERLOAD

TGF-β is the main stimulus for phosphorylation of SMAD2/3. GDF-15 has been demonstrated to have antihypertrophic and cardioprotective effects, possibly mediated by SMAD2/3 activation (19). SMAD2 activation has been reported in failing human hearts (66). Thus it remains unclear whether this signalling system mediates or counteracts cardiac dysfunction. SM16 is a small molecule inhibitor of ALK4 and 5 which leads to reduced phosphorylation of SMAD2/3. SM16 has been tested against 60 different kinases and except inhibiting ALK5 and ALK4, only weak inhibitory activity against P38 and Raf were demonstrated (64). In paper III

we have used SM16 to inhibit SMAD phosphorylation in mice subjected to aortic banding. We found that phosphorylation of SMAD2 was increased following aortic banding. Neither phosphorylation of P38, Raf nor SMAD3 was increased. Phosphorylation of SMAD2 was attenuated by SM16 treatment. SM16 preserved cardiac function, as evaluated by echocardiography and as the pulmonary congestion seen after aortic banding was abolished. Although SM16 led to significant inhibition of collagen gene transcription, myocardial collagen protein was not altered after one week of aortic banding. This observation makes potential effects on collagen synthesis a less likely explanation for the preservation of cardiac function demonstrated in our study. Aortic banding led to reduced left ventricular levels of SERCA2 (76, 77), while SM16 attenuated this SERCA2 loss. In studies of isolated cardiomyocytes, cells from SM16 treated mice demonstrated a more rapid decline of Ca^{2+} transients possibly due to improved SERCA2 function. Moreover, TGF-β1 stimulated neonatal rat cardiomyocytes demonstrated increased phosphorylation of SMAD2 and downregulation of SERCA2 that was inhibited by SM16. We suggest in paper III that SMAD signalling has effects in cardiomyocytes subjected to pressure overload and this finding might represent the first step to develop a pharmaceutical strategy to improve cardiac function by SMAD2 inhibition.

REVERSE REMODELING

Following aortic valve replacement for aortic stenosis, reverse remodelling of the left ventricle takes place. Left ventricular mass usually normalizes during the first 18 months subsequently followed by regression of fibrosis (14). Incomplete reverse remodelling is associated with persisting cardiac dysfunction (12). Knowledge about the reverse remodelling process is scarce and we do not know how this process is regulated. In paper IV we have studied reverse remodelling of the left ventricle in a mouse model of reversible pressure overload, described in detail in paper II. We identified a previously unknown collagen isoform shift during the early phase of reverse remodelling, as collagen type I was transiently increased by 50% at 3 days followed by a later increase in type III (50%) and VIII (70%) at 7 days after debanding. Collagen types I and III have different biomechanical properties. They are both fibrillar collagens, type I is the stiffer collagen being the dominant collagen in the Achilles tendon, while extensile properties has been attributed to collagen type III (33, 35). Collagen type VIII is a non-fibrillar collagen and little is known with respect to its biomechanical properties. Collagen type VIII is not normally expressed in the human heart, but involvement during embryologic development of the heart has been suggested (78). Collagen type VIII has been reported to be involved in tissue repair (79) and a role in vascular smooth muscle cell migration and MMP expression has been described (80, 81). We suggest that the collagen isoform shift observed during reverse remodelling might be

important for the biomechanical properties of the heart and the recovery of cardiac function following surgical correction of left ventricular pressure overload.

CONCLUSIONS

PAPER I:

We have observed an increase in plasma levels of activin A in patients scheduled for aortic valve replacement for aortic stenosis compared to healthy controls. Following aortic valve replacement there was a further increase at six and twelve months postoperatively with higher levels in symptomatic patients. Two days postoperatively circulating GDF-15, MMP-3 and -9 were increased and GDF-15 was correlated to MMP-3, which again correlated to left ventricular end diastolic diameter.

PAPER II:

We have established a hemodynamically well characterized mouse model of reversible left ventricular pressure overload by banding-debanding of the ascending aorta, inducing reverse myocardial remodelling.

PAPER III:

Pharmacological inhibition of SMAD2 signalling by SM16 during pressure overload in mice demonstrated that SM16 preserves cardiac function, possibly via beneficial effects on cardiomyocyte Ca^{2+} handling. Even though collagen mRNA expression was attenuated by SM16, myocardial collagen protein levels were not altered after one week of aortic banding.

PAPER IV:

Alterations in collagen gene expression were the most evident transcriptional changes during the early phase of reverse remodelling in mice. Even though total collagen protein remained increased following debanding, there was a subtype change as collagen type I increased transiently at 3 days followed by a later increase in type III and VIII at 7 days after debanding. We suggest that this collagen subtype shift during the early phase of reverse remodelling might be important for the biomechanical properties of the heart and the recovery of cardiac function.

PERSPECTIVES

The myocardial remodelling process seen in aortic stenosis eventually leads to cardiac dysfunction. Extensive remodelling may contribute to low cardiac output syndrome following aortic valve replacement, for which there is currently no effective treatment available. Incomplete reverse remodelling following aortic valve replacement for aortic stenosis is associated with persisting symptoms and increased mortality. Increased knowledge of the mechanisms regulating myocardial remodelling and cardiac function, such as the possible effect of TGF-β/SMAD signalling on cardiomyocyte SERCA2 levels and Ca^{2+} homeostasis, might lead to new pharmacologic strategies in the treatment of heart failure. Understanding the reverse remodelling process following correction of left ventricular pressure overload might lead to treatments enhancing this process. This may be beneficial if reverse remodelling following aortic valve replacement for aortic stenosis is incomplete and possibly also for patients with hypertensive heart failure.

To gain further knowledge it is the ambition of our research group to do further studies on these topics. The possible role of collagen VIII during reverse remodelling is intriguing. Both direct mechanical effects and effects from interaction with other proteins in the ECM are possible. A collagen VIII knock-out has been developed (82, 83) and is available to our group. It is our ambition to use this knock-out mouse to address the possible role of collagen type VIII in remodelling and reverse remodelling.

REFERENCE LIST

1. Norsk Thoraxkirurgisk Forening. Hjertekirurgiregisteret. Norsk Thoraxkirurgisk Forening. www.legeforeningen.no/thorax.

2. Bonow RO, Carabello BA, Kanu C, de Leon ACJ, Faxon DP, Freed MD, Gaasch WH, Lytle BW, Nishimura RA, O'Gara PT, O'Rourke RA, Otto CM, Shah PM, Shanewise JS, Smith SC, Jr., Jacobs AK, Adams CD, Anderson JL, Antman EM, Faxon DP, Fuster V, Halperin JL, Hiratzka LF, Hunt SA, Lytle BW, Nishimura R, Page RL, Riegel B. ACC/AHA 2006 guidelines for the management of patients with valvular heart disease: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (writing committee to revise the 1998 Guidelines for the Management of Patients With Valvular Heart Disease): developed in collaboration with the Society of Cardiovascular Anesthesiologists: endorsed by the Society for Cardiovascular Angiography and Interventions and the Society of Thoracic Surgeons. *Circulation* 2006;114:e84-231.

3. Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA. Controversies in ventricular remodelling. *Lancet* 2006;367:356-367.

4. Schoenfeld JR, Vasser M, Jhurani P, Ng P, Hunter JJ, Ross J, Jr., Chien KR, Lowe DG. Distinct molecular phenotypes in murine cardiac muscle development, growth, and hypertrophy. *J Mol Cell Cardiol* 1998;30:2269-2280.

5. van den Bosch BJ, Lindsey PJ, van den Burg CM, van der Vlies SA, Lips DJ, van der Vusse GJ, Ayoubi TA, Doevendans PA, Smeets HJ. Early and transient gene expression changes in pressure overload-induced cardiac hypertrophy in mice. *Genomics* 2006;88:480-488.

6. McMullen JR, Jennings GL. Differences between pathological and physiological cardiac hypertrophy: novel therapeutic strategies to treat heart failure. *Clin Exp Pharmacol Physiol* 2007;34:255-262.

7. Depre C, Shipley GL, Chen W, Han Q, Doenst T, Moore ML, Stepkowski S, Davies PJ, Taegtmeyer H. Unloaded heart in vivo replicates fetal gene expression of cardiac hypertrophy. *Nat Med* 1998;4:1269-1275.

8. Kupari M, Turto H, Lommi J. Left ventricular hypertrophy in aortic valve stenosis: preventive or promotive of systolic dysfunction and heart failure? *Eur Heart J* 2005;26:1790-1796.

9. Ross J, Jr., Braunwald E. Aortic stenosis. *Circulation* 1968;38:61-67.

10. Iung B. Management of the elderly patient with aortic stenosis. *Heart* 2008;94:519-524.

11. Wendt D, Osswald B, Thielmann M, Kayser K, Tossios P, Massoudy P, Kamler M, Jakob H. The EuroSC. *Interact Cardiovasc Thorac Surg* 2010;10:239-244.

12. Lund O, Kristensen LH, Baandrup U, Hansen OK, Nielsen TT, Emmertsen K, Jensen FT, Flo C, Rasmussen BS, Pilegaard HK. Myocardial structure as a determinant of pre- and postoperative ventricular function and long-term prognosis after valve replacement for aortic stenosis. *Eur Heart J* 1998;19:1099-1108.

13. Perez de AD, Lees B, Flather M, Nugara F, Husebye T, Jasinski M, Cisowski M, Khan M, Henein M, Gaer J, Guvendik L, Bochenek A, Wos S, Lie M, Van NG, Pennell D, Pepper J. Randomized comparison of stentless versus stented valves for aortic stenosis: effects on left ventricular mass. *Circulation* 2005;112:2696-2702.

14. Krayenbuehl HP, Hess OM, Monrad ES, Schneider J, Mall G, Turina M. Left ventricular myocardial structure in aortic valve disease before, intermediate, and late after aortic valve replacement. *Circulation* 1989;79:744-755.

15. Tønnessen T, Giaid A, Saleh D, Næss PA, Yanagisawa M, Christensen G. Increased in vivo expression and production of endothelin-1 by porcine cardiomyocytes subjected to ischemia. *Circ Res* 1995;76:767-772.

16. Woldbæk PR, Tønnessen T, Henriksen UL, Florholmen G, Lunde PK, Lyberg T, Christensen G. Increased cardiac IL-18 mRNA, pro-IL-18 and plasma IL-18 after myocardial infarction in the mouse; a potential role in cardiac dysfunction. *Cardiovasc Res* 2003;59:122-131.

17. Woldbæk PR, Sande JB, Strømme TA, Lunde PK, Djurovic S, Lyberg T, Christensen G, Tønnessen T. Daily administration of interleukin-18 causes myocardial dysfunction in healthy mice. *Am J Physiol Heart Circ Physiol* 2005;289:H708-H714.

18. Khan R, Sheppard R. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. *Immunology* 2006;118:10-24.

19. Xu J, Kimball TR, Lorenz JN, Brown DA, Bauskin AR, Klevitsky R, Hewett TE, Breit SN, Molkentin JD. GDF15/MIC-1 functions as a protective and antihypertrophic factor released from the myocardium in association with SMAD protein activation. *Circ Res* 2006;98:342-350.

20. Rosenkranz S, Flesch M, Amann K, Haeuseler C, Kilter H, Seeland U, Schluter KD, Bohm M. Alterations of beta-adrenergic signaling and cardiac hypertrophy in transgenic mice overexpressing TGF-beta(1). *Am J Physiol Heart Circ Physiol* 2002;283:H1253-H1262.

21. Florholmen G, Halvorsen B, Beraki K, Lyberg T, Sagen EL, Aukrust P, Christensen G, Yndestad A. Activin A inhibits organization of sarcomeric proteins in cardiomyocytes induced by leukemia inhibitory factor. *J Mol Cell Cardiol* 2006;41:689-697.

22. Yndestad A, Ueland T, Øie E, Florholmen G, Halvorsen B, Attramadal H, Simonsen S, Frøland SS, Gullestad L, Christensen G, Damås JK, Aukrust P. Elevated levels of activin A in heart failure: potential role in myocardial remodeling. *Circulation* 2004;109:1379-1385.

23. Schiller M, Javelaud D, Mauviel A. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 2004;35:83-92.

24. Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 2007;8:970-982.

25. Euler-Taimor G, Heger J. The complex pattern of SMAD signaling in the cardiovascular system. *Cardiovasc Res* 2006;69:15-25.

26. Wrana JL, Attisano L. The Smad pathway. *Cytokine Growth Factor Rev* 2000;11:5-13.

27. Eghbali M, Weber KT. Collagen and the myocardium: fibrillar structure, biosynthesis and degradation in relation to hypertrophy and its regression. *Mol Cell Biochem* 1990;96:1-14.

28. Jalil JE, Doering CW, Janicki JS, Pick R, Shroff SG, Weber KT. Fibrillar collagen and myocardial stiffness in the intact hypertrophied rat left ventricle. *Circ Res* 1989;64:1041-1050.

29. Corda S, Samuel JL, Rappaport L. Extracellular matrix and growth factors during heart growth. *Heart Fail Rev* 2000;5:119-130.

30. Caulfield JB, Janicki JS. Structure and function of myocardial fibrillar collagen. *Technol Health Care* 1997;5:95-113.

31. Yurchenco PD, Schittny JC. Molecular architecture of basement membranes. *FASEB J* 1990;4:1577-1590.

32. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 1991;83:1849-1865.

33. O'Brien M. The anatomy of the Achilles tendon. *Foot Ankle Clin* 2005;10:225-238.

34. Lodish H, Berk A, Kaiser CA, Krieger M, Scott MP, Bretscher A, Ploegh H, Matsudaira PT. The Extracellular Matrix II: Connective and Other Tissues. *Molecular cell biology*. 6th ed. ed. New York: Freeman; 2008. p. 825-833.

35. Alexander JJ. The pathobiology of aortic aneurysms. *J Surg Res* 2004;117:163-175.

36. Liapis A, Bakas P, Pafiti A, Frangos-Plemenos M, Arnoyannaki N, Creatsas G. Changes of collagen type III in female patients with genuine stress incontinence and pelvic floor prolapse. *Eur J Obstet Gynecol Reprod Biol* 2001;97:76-79.

37. Wegrowski Y, Bellon G, Quereux C, Maquart FX. Biochemical alterations of uterine leiomyoma extracellular matrix in type IV Ehlers-Danlos syndrome. *Am J Obstet Gynecol* 1999;180:1032-1034.

38. Heino J. The collagen family members as cell adhesion proteins. *Bioessays* 2007;29:1001-1010.

39. Spinale FG. Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. *Physiol Rev* 2007;87:1285-1342.

40. Lucas JA, Zhang Y, Li P, Gong K, Miller AP, Hassan E, Hage F, Xing D, Wells B, Oparil S, Chen YF. Inhibition of transforming growth factor-beta signaling induces left ventricular dilation and dysfunction in the pressure-overloaded heart. *Am J Physiol Heart Circ Physiol* 2010;298:H424-H432.

41. Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K, Imaizumi T. Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. *Circulation* 2002;106:130-135.

42. Duncan AI, Lowe BS, Garcia MJ, Xu M, Gillinov AM, Mihaljevic T, Koch CG. Influence of concentric left ventricular remodeling on early mortality after aortic valve replacement. *Ann Thorac Surg* 2008;85:2030-2039.

43. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, Attwood J, Baertsch R, Bailey J, Barlow K, Beck S, Berry E, Birren B, Bloom T, Bork P, Botcherby M, Bray N, Brent MR, Brown DG, Brown SD, Bult C, Burton J, Butler J, Campbell RD, Carninci P, Cawley S, Chiaromonte F, Chinwalla AT, Church DM, Clamp M, Clee C, Collins FS, Cook LL, Copley RR, Coulson A, Couronne O, Cuff J, Curwen V, Cutts T, Daly M, David R, Davies J, Delehaunty KD, Deri J, Dermitzakis ET, Dewey C, Dickens NJ, Diekhans M, Dodge S, Dubchak I, Dunn DM, Eddy SR, Elnitski L, Emes RD, Eswara P, Eyras E, Felsenfeld A, Fewell GA, Flicek P, Foley K, Frankel WN, Fulton LA, Fulton RS, Furey TS, Gage D, Gibbs RA, Glusman G, Gnerre S, Goldman N, Goodstadt L, Grafham D, Graves TA, Green ED, Gregory S, Guigo R, Guyer M, Hardison RC, Haussler D, Hayashizaki Y, Hillier LW, Hinrichs A, Hlavina W, Holzer T, Hsu F, Hua A, Hubbard T, Hunt A, Jackson I, Jaffe DB, Johnson LS, Jones M, Jones TA, Joy A, Kamal M, Karlsson EK, Karolchik D, Kasprzyk A, Kawai J, Keibler E, Kells C, Kent WJ, Kirby A, Kolbe DL, Korf I, Kucherlapati RS, Kulbokas EJ, Kulp D, Landers T, Leger JP, Leonard S, Letunic I, Levine R, Li J, Li M, Lloyd C, Lucas S, Ma B, Maglott DR, Mardis

ER, Matthews L, Mauceli E, Mayer JH, McCarthy M, McCombie WR, McLaren S, McLay K, McPherson JD, Meldrim J, Meredith B, Mesirov JP, Miller W, Miner TL, Mongin E, Montgomery KT, Morgan M, Mott R, Mullikin JC, Muzny DM, Nash WE, Nelson JO, Nhan MN, Nicol R, Ning Z, Nusbaum C, O'Connor MJ, Okazaki Y, Oliver K, Overton-Larty E, Pachter L, Parra G, Pepin KH, Peterson J, Pevzner P, Plumb R, Pohl CS, Poliakov A, Ponce TC, Ponting CP, Potter S, Quail M, Reymond A, Roe BA, Roskin KM, Rubin EM, Rust AG, Santos R, Sapojnikov V, Schultz B, Schultz J, Schwartz MS, Schwartz S, Scott C, Seaman S, Searle S, Sharpe T, Sheridan A, Shownkeen R, Sims S, Singer JB, Slater G, Smit A, Smith DR, Spencer B, Stabenau A, Stange-Thomann N, Sugnet C, Suyama M, Tesler G, Thompson J, Torrents D, Trevaskis E, Tromp J, Ucla C, Ureta-Vidal A, Vinson JP, Von Niederhausern AC, Wade CM, Wall M, Weber RJ, Weiss RB, Wendl MC, West AP, Wetterstrand K, Wheeler R, Whelan S, Wierzbowski J, Willey D, Williams S, Wilson RK, Winter E, Worley KC, Wyman D, Yang S, Yang SP, Zdobnov EM, Zody MC, Lander ES. Initial sequencing and comparative analysis of the mouse genome. *Nature* 2002;420:520-562.

44. Rottman JN, Ni G, Brown M. Echocardiographic evaluation of ventricular function in mice. *Echocardiography* 2007;24:83-89.

45. Cahan P, Li Y, Izumi M, Graubert TA. The impact of copy number variation on local gene expression in mouse hematopoietic stem and progenitor cells. *Nat Genet* 2009;41:430-437.

46. Tarnavski O. Mouse surgical models in cardiovascular research. *Methods Mol Biol* 2009;573:115-137.

47. Finsen AV, Christensen G, Sjaastad I. Echocardiographic parameters discriminating myocardial infarction with pulmonary congestion from myocardial infarction without congestion in the mouse. *J Appl Physiol* 2005;98:680-689.

48. Alexa A, Rahnenfuhrer J, Lengauer T. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics* 2006;22:1600-1607.

49. Benjamini Y, Hochberg Y. Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J Roy Statist Soc Ser B* 1995;1:289-300.

50. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986-994.

51. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* 2008;44:619-626.

52. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat Protocols* 2006;1:1559-1582.

53. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350-4354.

54. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981;112:195-203.

55. Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin Chem* 2005;51:2415-2418.

56. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 1971;8:871-874.

57. Kofoed K, Schneider UV, Scheel T, Andersen O, Eugen-Olsen J. Development and validation of a multiplex add-on assay for sepsis biomarkers using xMAP technology. *Clin Chem* 2006;52:1284-1293.

58. Chergui F, Chretien AS, Bouali S, Ramacci C, Rouyer M, Bastogne T, Genin P, Leroux A, Merlin JL. Validation of a phosphoprotein array assay for characterization of human tyrosine kinase receptor downstream signaling in breast cancer. *Clin Chem* 2009;55:1327-1336.

59. Issaq HJ, Chan KC, Blonder J, Ye X, Veenstra TD. Separation, detection and quantitation of peptides by liquid chromatography and capillary electrochromatography. *J Chromatogr A* 2009;1216:1825-1837.

60. Louch WE, Mørk HK, Sexton J, Strømme TA, Laake P, Sjaastad I, Sejersted OM. T-tubule disorganization and reduced synchrony of Ca^{2+} release in murine cardiomyocytes following myocardial infarction. *J Physiol* 2006;574:519-533.

61. Holt E, Christensen G. Transient Ca^{2+} overload alters Ca^{2+} handling in rat cardiomyocytes: effects on shortening and relaxation. *Am J Physiol* 1997;273:H573-H582.

62. Sande JB, Sjaastad I, Hoen IB, Bøkenes J, Tønnessen T, Holt E, Lunde PK, Christensen G. Reduced level of serine(16) phosphorylated phospholamban in the failing rat myocardium: a major contributor to reduced SERCA2 activity. *Cardiovasc Res* 2002;53:382-391.

63. Florholmen G, Andersson KB, Yndestad A, Austbo B, Henriksen UL, Christensen G. Leukaemia inhibitory factor alters expression of genes involved in rat cardiomyocyte energy metabolism. *Acta Physiol Scand* 2004;180:133-142.

64. Fu K, Corbley MJ, Sun L, Friedman JE, Shan F, Papadatos JL, Costa D, Lutterodt F, Sweigard H, Bowes S, Choi M, Boriack-Sjodin PA, Arduini RM, Sun D, Newman MN, Zhang X, Mead JN,

Chuaqui CE, Cheung HK, Zhang X, Cornebise M, Carter MB, Josiah S, Singh J, Lee WC, Gill A, Ling LE. SM16, an orally active TGF-beta type I receptor inhibitor prevents myofibroblast induction and vascular fibrosis in the rat carotid injury model. *Arterioscler Thromb Vasc Biol* 2008;28:665-671.

65. Suzuki E, Kim S, Cheung HK, Corbley MJ, Zhang X, Sun L, Shan F, Singh J, Lee WC, Albelda SM, Ling LE. A novel small-molecule inhibitor of transforming growth factor beta type I receptor kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection. *Cancer Res* 2007;67:2351-2359.

66. Jahanyar J, Joyce DL, Southard RE, Loebe M, Noon GP, Koerner MM, Torre-Amione G, Youker KA. Decorin-mediated transforming growth factor-beta inhibition ameliorates adverse cardiac remodeling. *J Heart Lung Transplant* 2007;26:34-40.

67. Churchill GA. Fundamentals of experimental design for cDNA microarrays. *Nat Genet* 2002;32 Suppl:490-495.

68. Liao Y, Ishikura F, Beppu S, Asakura M, Takashima S, Asanuma H, Sanada S, Kim J, Ogita H, Kuzuya T, Node K, Kitakaze M, Hori M. Echocardiographic assessment of LV hypertrophy and function in aortic-banded mice: necropsy validation. *Am J Physiol Heart Circ Physiol* 2002;282:H1703-H1708.

69. Peng Y, Popovic ZB, Sopko N, Drinko J, Zhang Z, Thomas JD, Penn MS. Speckle tracking echocardiography in the assessment of mouse models of cardiac dysfunction. *Am J Physiol Heart Circ Physiol* 2009;297:H811-H820.

70. Itoh S, Itoh F, Goumans MJ, Ten DP. Signaling of transforming growth factor-beta family members through Smad proteins. *Eur J Biochem* 2000;267:6954-6967.

71. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577-584.

72. Wang B, Hao J, Jones SC, Yee MS, Roth JC, Dixon IM. Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart. *Am J Physiol Heart Circ Physiol* 2002;282:H1685-H1696.

73. Wang B, Omar A, Angelovska T, Drobic V, Rattan SG, Jones SC, Dixon IM. Regulation of collagen synthesis by inhibitory Smad7 in cardiac myofibroblasts. *Am J Physiol Heart Circ Physiol* 2007;293:H1282-H1290.

74. Sakata Y, Chancey AL, Divakaran VG, Sekiguchi K, Sivasubramanian N, Mann DL. Transforming growth factor-beta receptor antagonism attenuates myocardial fibrosis in mice with cardiac-restricted overexpression of tumor necrosis factor. *Basic Res Cardiol* 2008;103:60-68.

75. Bujak M, Frangogiannis NG. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. *Cardiovasc Res* 2007;74:184-195.

76. de la Bastie D, Levitsky D, Rappaport L, Mercadier JJ, Marotte F, Wisnewsky C, Brovkovich V, Schwartz K, Lompre AM. Function of the sarcoplasmic reticulum and expression of its Ca2(+)-ATPase gene in pressure overload-induced cardiac hypertrophy in the rat. *Circ Res* 1990;66:554-564.

77. Levitsky D, de la Bastie D, Schwartz K, Lompre AM. Ca(2+)-ATPase and function of sarcoplasmic reticulum during cardiac hypertrophy. *Am J Physiol* 1991;261:23-26.

78. Iruela-Arispe ML, Sage EH. Expression of type VIII collagen during morphogenesis of the chicken and mouse heart. *Dev Biol* 1991;144:107-118.

79. Shuttleworth CA. Type VIII collagen. *Int J Biochem Cell Biol* 1997;29:1145-1148.

80. Hou G, Mulholland D, Gronska MA, Bendeck MP. Type VIII collagen stimulates smooth muscle cell migration and matrix metalloproteinase synthesis after arterial injury. *Am J Pathol* 2000;156:467-476.

81. Cherepanova OA, Pidkovka NA, Sarmento OF, Yoshida T, Gan Q, Adiguzel E, Bendeck MP, Berliner J, Leitinger N, Owens GK. Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circ Res* 2009;104:609-618.

82. Hopfer U, Fukai N, Hopfer H, Wolf G, Joyce N, Li E, Olsen BR. Targeted disruption of Col8a1 and Col8a2 genes in mice leads to anterior segment abnormalities in the eye. *FASEB J* 2005;19:1232-1244.

83. Hopfer U, Hopfer H, Meyer-Schwesinger C, Loeffler I, Fukai N, Olsen BR, Stahl RA, Wolf G. Lack of type VIII collagen in mice ameliorates diabetic nephropathy. *Diabetes* 2009;58:1672-1681.

ERRATA

Page 22, legend figure 6: “A: n=13-108” should have been “A: n=8-28”

Page 29, line 13: “mice” should have been “mouse”

I

II

III

IV